3 SEMEN IDENTIFICATION

3.1 ACID PHOSPHATASE TEST (Reference 5, pp. 162-163, Appendix A)

3.1.1 Safety Considerations

3.1.1.1 Glacial acetic acid - Caution! Corrosive! Flammable!

3.1.1.2 Sodium acetate - Caution! Irritant!

3.1.1.3 Sodium α-naphthyl acid phosphate - Caution! Irritant! Emits toxic fumes under fire conditions!

3.1.1.4 o-Dianisidine (Naphthanil diazo blue B) - Caution! Highly toxic! Emits toxic fumes under fire conditions!

3.1.1.5 Naphthanil diazo red - Caution! Avoid contact and inhalation! Emits toxic fumes under fire conditions!

3.1.2 Equipment

3.1.2.1 5 ml and 500 ml Graduated cylinders

3.1.2.2 Balance

3.1.2.3 Spatula

3.1.2.4 Scissors

3.1.2.5 Tweezers

3.1.3 Materials

3.1.3.1 Filter paper or microtiter plate (optional)

3.1.3.2 Weigh boats or weigh paper

3.1.3.3 Cotton swabs

3.1.3.4 Test tubes or bottles

3.1.3.5 Disposable transfer pipets or droppers

3.1.4 Working Solutions

3.1.4.1 Acid Phosphatase (AP) Buffer

- 2.5 ml Glacial acetic acid
- 10.0 g Sodium acetate (anhydrous)
- 450.0 ml Distilled water
- Mix the above ingredients until thoroughly dissolved.

3.1.4.1.1 Storage

3.1.4.1.1.1 The AP Buffer is stable at room temperature.

3.1.4.1.2 Labeling

3.1.4.1.2.1 Label the bottle as AP Buffer with a lot number (the date of preparation followed by the initials of the person preparing the stock solution).
Example: AP Buffer Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

3.1.4.1.2.2 There is no expiration date (see 3.1.5 Minimum Standards and Controls).

3.1.4.2 Sodium α-Naphthyl Acid Phosphate Solution

3.1.4.2.1 Add a small amount (approximately 4 mg) of sodium α-naphthyl acid phosphate to approximately 3 ml of Acid Phosphatase buffer in an appropriately labeled 10 X 75 mm test tube or bottle.

3.1.4.2.2 Discard the solution at the end of the day.

3.1.4.3 Dye Solution

3.1.4.3.1 Add a small amount (approximately 4 mg) of o-dianisidine or naphthanil diazo red to approximately 3 ml of buffer in an appropriately labeled 10 X 75 mm test tube or bottle.

3.1.4.3.2 Discard the solution at the end of the day.

3.1.4.4 Distilled water

3.1.5 Minimum Standards and Controls

3.1.5.1 On the day of use a positive reagent control (known semen stain) and a negative reagent control (distilled water) must be tested to ensure that the reagents are working properly. The results of this testing must be documented in the case file.

3.1.5.2 If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved as demonstrated by testing another set of positive and negative reagent controls and achieving the expected results with both controls.

3.1.5.3 If the results of the test are positive, a substrate control (if available) must also be tested, unless the stain is on a cotton swab, and the results of the testing documented in the case file. It is not necessary to test submitted control swabs.

3.1.6 ACID PHOSPHATASE (AP) TEST PROCEDURE (Sodium α-Napthyl Acid Phosphate)

3.1.6.1 Moisten filter paper/swab with distilled water. (Do not use buffer solution, as this will contaminate the stained area.) Press the filter paper against the suspected stain or gently rub the stained area with the moistened swab. Alternatively, a small piece of the stain/swab can be placed on filter paper, in a small test tube, or in a microtiter plate. Treat the substrate control in the same manner.

3.1.6.2 Add 1-2 drops of sodium α-naphthyl acid phosphate solution.

3.1.6.3 Add 1-2 drops of dye solution.

3.1.6.4 The development of a blue/purple color with o-dianisidine or an orange/red color with naphthanil diazo red within 10 to 15 seconds is indicative of acid phosphatase levels in the semen range.

3.1.6.5 The presence of semen in all samples exhibiting an inconclusive result or a positive result must be confirmed by identifying spermatozoa or, in the absence of spermatozoa, p30.

3.1.6.6 Interpretation

3.1.6.6.1 Positive Reaction = Blue/purple color with o-dianisidine within 10 to 15 seconds

OR

Orange/red color with naphthanil diazo red within 10 to 15 seconds

3.1.6.6.2 Negative Reaction = No color development, slight/slow color development

3.1.6.6.3 Inconclusive Reaction = Slow moderate to strong color development

3.1.6.7 Refer to Section 3.8 for reporting results.

3.2 EXTRACTION OF SPERMATOZOA FROM A SUBSTRATE

3.2.1 Equipment

3.2.1.1 Rotator, vortex, sonicator,or centrifuge (depending on extraction method used)

3.2.1.2 Scissors

3.2.1.3 Tweezers

3.2.1.4 Dissecting needle (optional)

3.2.2 Materials

3.2.2.1 Microscope slides

3.2.2.2 Test tubes

3.2.3 Reagents

3.2.3.1 Distilled water

3.2.4 Extraction Methods

3.2.4.1 Cut a small portion of a stain and soak in a test tube overnight in distilled water.

3.2.4.2 Soak a small portion of a stain in distilled water and rotate overnight.

3.2.4.3 Soak a small portion of a stain in distilled water and sonicate for 10 seconds, followed by a 30 second sonication.

3.2.4.4 Tease fibers apart and soak in a small amount of distilled water.

3.2.4.5 Soak a small portion of a stain in distilled water and vortex.

3.2.4.6 Soak a small portion of a stain in distilled water on a microscope slide, stain side down (may be followed by mastication).

3.2.4.7 Cut the stain into small pieces, place the pieces on a microscope slide, and soak in a small amount of distilled water (may be followed by mastication).

3.2.4.8 For the OneStep ABAcard® p30 Test extraction method, refer to 3.7.9.1 through 3.7.9.7.

NOTES: Always soak the material first; prolong the soaking for difficult stains.
Use the sonicator on low (high setting will disintegrate spermatozoa).
To concentrate an extract, after soaking a small portion of a stain or swab, centrifuge and make a smear of the sediment.

DNA extracts can also be used to search for spermatozoa.

3.3 KERNECHTROT-PICROINDIGOCARMINE STAIN (CHRISTMAS TREE STAIN) (Reference 7, p. 141, Appendix A)

3.3.1 Kernechtrot-Picroindigocarmine (KPIC) differential biological stain is used to assist in the microscopic identification of spermatozoa. The solutions for this procedure can either be purchased (SERI) or prepared in-house.

3.3.2 Safety Considerations

3.3.2.1 Aluminum sulfate - Caution! Harmful if inhaled, in contact with skin, and if swallowed! Emits toxic fumes under fire conditions!

3.3.2.2 Nuclear fast red - Caution! Irritant! Emits toxic fumes under fire conditions!

3.3.2.3 Saturated picric acid solution - Caution! Toxic! Explosive when dry! Emits toxic fumes under fire conditions!

3.3.2.4 Indigocarmine dye - Caution! Harmful if swallowed! Emits toxic fumes under fire conditions!

3.3.3 Equipment

3.3.3.1 Flame or heat block

3.3.4 Materials

3.3.4.1 Fixative (optional)

3.3.5 Reagents

3.3.5.1 Kernechtrot staining solution (KS)

3.3.5.2 Picroindigocarmine staining solution (PICS)

3.3.5.3 Distilled water

3.3.5.4 95% ethanol or methanol

3.3.6 Stock Solutions (In-house Preparation)

3.3.6.1 Equipment

3.3.6.1.1 Filtration apparatus

3.3.6.1.2 500 ml glass beakers

3.3.6.1.3 Balance

3.3.6.1.4 Spatula

3.3.6.1.5 Glass rod

3.3.6.1.6 Plastic bottles

3.3.6.2 Materials

3.3.6.2.1 Filter paper

3.3.6.2.2 Weigh boats or weigh paper

3.3.6.3 Reagents

3.3.6.3.1 Aluminum sulfate

3.3.6.3.2 Nuclear Fast Red

3.3.6.3.3 Distilled water

3.3.6.3.4 Picroindigocarmine dye

3.3.6.3.5 Saturated picric acid solution **(Purchase saturated solution. DO NOT PURCHASE DRY PRODUCT! See 3.3.2.3 Safety Considerations.)**

3.3.6.4 Kernechtrot Solution (KS)

- In a beaker dissolve 5 g of aluminum sulfate in 100 ml of hot distilled water.
- Immediately add 0.1 g of Nuclear Fast Red and stir with a glass rod.
- Allow to cool and filter through filter paper.

3.3.6.4.1 Storage

3.3.6.4.1.1 The Kernechtrot Solution is stable at room temperature for up to 6 months, but may need to be refiltered after standing.

3.3.6.4.2 Labeling

3.3.6.4.2.1 Label the bottle as KS with the expiration date and a lot number (the date of preparation followed by the initials of person preparing the solution).
Example: KS Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

3.3.6.5 Picroindigocarmine Solution (PICS)

- Dissolve 1 g of Indigocarmine dye in 300 ml of a commercially purchased saturated solution of picric acid.
- Filter and store.

3.3.6.5.1 Storage

3.3.6.5.1.1 The Picroindigocarmine Solution is stable at room temperature for up to 6 months, but may need to be refiltered after standing.

3.3.6.5.2 Labeling

3.3.6.5.2.1 Label the bottle as PICS with an expiration date and a lot number (the date of preparation followed by the initials of person preparing the solution).
Example: PICS Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

3.3.7 SERI Christmas Tree Stain (R540) Kit

3.3.7.1 Contents

3.3.7.1.1 Solution A (Kernechtrot Solution - KS) - 30 ml

3.3.7.1.2 Solution B (Picroindigocarmine Solution - PICS) - 30 ml

3.3.7.1.3 Directions for use.

3.3.7.2 Store under refrigeration in bottles provided.

3.3.7.3 Shelf life: 6 months

3.3.8 KPICS/CHRISTMAS TREE STAINING PROCEDURE

3.3.8.1 Prepare a thin smear of an extract of a suspected semen stain and allow to dry, or use a smear from the Physical Evidence Recovery Kit (PERK). Fix the smear with a quick flame or fixative, or by placing it on a heat block overnight.

3.3.8.2 Add a sufficient amount (2-5 drops) of KS (red reagent) to cover the stained portion of the microscope slide.

3.3.8.3 Let the slide stand at room temperature for at least 15 minutes.

3.3.8.4 Wash KS off of the slide with a gentle stream of distilled water and drain the slide.

3.3.8.5 Add a sufficient amount (2-5 drops) of PICS (green reagent) to cover the stained portion of the slide.

3.3.8.6 Allow PICS to stain the smear for 5-15 seconds.

3.3.8.7 Wash PICS off of the slide with 95% ethanol or methanol.

3.3.8.8 Dry the slide at room temperature.

3.4 PROCEDURE FOR KÖEHLER ILLUMINATION (Reference 8, Appendix A)

3.4.1 Determine that the lamp is centered according to the instructions for the microscope in use.

3.4.2 Using a medium to low power objective (approximately 10X), place a specimen in position and focus.

3.4.3 Close the field diaphragm.

3.4.4 Focus the image of the field diaphragm by adjusting the substage condenser.

3.4.5 Center the field diaphragm using the centering screws on the condenser.

3.4.6 Open the field diaphragm so that the rim just disappears beyond the field of view.

3.4.7 Adjust the condenser diaphragm (aperture diaphragm) to about ½ of the full aperture.

NOTE: Resolution, contrast, and depth of field can be regulated with the condenser diaphragm. It should not be used to regulate the brightness. For this purpose, either the regulating transformer or neutral density filters should be used.

3.5 MICROSCOPIC EXAMINATION OF STAINED SLIDES FOR SPERMATOZOA

3.5.1 Equipment

3.5.1.1 Microscope (with approximately 200X – 400X total magnification, with or without phase capability)

3.5.2 Materials

3.5.2.1 Distilled water, xylene substitute, or other appropriate mounting medium

3.5.2.2 Coverslips

3.5.3 Procedure

3.5.3.1 Quickly scan at approximately 200X total magnification. Confirm at approximately 400X total magnification.

3.5.3.1.1 With phase microscopy: Spermatozoa heads are neon-like pink/red with darker pink/purple acrosomal caps and green tails. Epithelial cells and most bacteria stain green with some of the nuclei pink/red; however, these are shaped differently than spermatozoa. Yeast cells take on the same color as spermatozoa, but are shaped differently.

3.5.3.1.2 Without phase microscopy: Spermatozoa heads are neon-like pink/red with pale pink (almost colorless) acrosomal caps, blue-green necks/midpieces, and green tails. Epithelial cells appear bright blue with red to purple nuclei.

3.5.3.2 Document the approximate number of spermatozoa and spermatozoa heads on the smear per hpf (approximately 400X total magnification), per lpf (approximately 200X total magnification), per length of slide, or per slide, as appropriate. **If only 1 spermatozoon or spermatozoon head is observed, there must be documented confirmation of its presence by a second qualified examiner.**

3.5.3.3 Place all smears submitted in the PERK back into the PERK. Properly label and return all other spermatozoa positive smears with the evidence. **Note: If a stain is consumed in the preparation of a smear, properly label and return the smear even when no spermatozoa are identified.**

3.5.4 Refer to Section 3.8 for reporting results.

3.6 MICROSCOPIC EXAMINATION OF UNSTAINED SLIDES FOR SPERMATOZOA

3.6.1 Unstained smears may be examined using phase contrast microscopy.

3.6.2 Equipment

3.6.2.1 Microscope (approximately 200X – 400X total magnification) with phase capability

3.6.3 Materials

3.6.3.1 Microscope slides

3.6.3.2 Coverslips

3.6.3.3 Applicator sticks

3.6.4 Reagents

3.6.4.1 Distilled water

3.6.5 Procedure

3.6.5.1 Place a small amount of an extract of a suspected semen stain on a microscope slide and cover with a coverslip, or add a drop of distilled water to a smear from the PERK, use an applicator stick to mix the water and the material on the smear, and cover with a coverslip.

3.6.5.2 Scan quickly with phase at approximately 200X total magnification. Confirm with phase at approximately 400X total magnification.

3.6.5.3 When the coverslip is touched gently, the spermatozoa and/or spermatozoa heads will roll, exhibiting their characteristic 3-dimensional shape. Use the distinctive size and morphology to identify the spermatozoa/spermatozoa heads.

3.6.5.4 Document the approximate number of spermatozoa and spermatozoa heads on the smear per hpf (approximately 400X total magnification), per lpf (approximately 200X total magnification), per length of slide, or per slide, as appropriate. **If only 1 spermatozoon or spermatozoon head is observed, there must be documented confirmation of its presence by a second qualified examiner.**

3.6.5.5 Place all smears submitted in the PERK back into the PERK. Properly label and return all other spermatozoa positive smears. **Note: If a stain is consumed in the preparation of a smear, properly label and return the smear even when no spermatozoa are identified.**

3.6.5.6 Refer to Section 3.8 for reporting results.

3.7 P30 BY ONESTEP ABACARD® (References 9, 10, 11, Appendix A)

3.7.1 Quality Control

3.7.1.1 Before using a new lot number of the ABAcard® OneStep p30 Detection Test, its specificity must be tested and appropriately documented in the laboratory's quality control records.

3.7.1.2 The ABAcards® ("test devices") must be tested against human blood, vaginal fluid, saliva, feces, urine, a positive control (semen), and a negative control (distilled water) to ensure that the test is semen specific.

3.7.1.2.1 Samples of human blood, vaginal fluid, saliva, feces, urine, and semen will be prepared in-house.

3.7.1.2.2 Label the known samples with the name of the substance (i.e., human semen, etc.) and the lot number (the date of preparation followed by the initials of the person preparing the sample).
Example: human semen Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

3.7.1.2.3 Store known samples in the freezer.

3.7.1.2.4 It is also desirable to test dilutions of semen to determine the sensitivity of the test.

3.7.1.3 The quality control documentation will include:

3.7.1.3.1 The lot number, receipt date, expiration date, and manufacturer of the ABAcard® OneStep p30 Detection Test.

3.7.1.3.2 The date of testing.

3.7.1.3.3 Initials of the person conducting the testing.

3.7.1.3.4 Results of the testing.

3.7.1.4 Once the appropriate tests have been performed on a lot number of the ABAcard® OneStep p30 Detection Test, they need not be repeated for each case. If another shipment of the same lot number is received on a different date, the QC testing described above must be repeated.

3.7.2 "High Dose Hook Effect"

3.7.2.1 The "High Dose Hook Effect" is a false negative result that is obtained in the presence of high concentrations of p30 (usually undiluted semen). This effect results from large amounts of human p30 binding to the antibody to form an antigen-antibody complex and free p30 migrating toward the test area "T". The antibody in the test area "T" is blocked by this free p30. Therefore, the mobile antigen-antibody complex cannot bind to the antibody. As a result no pink line will form in the test area "T". To confirm the presence of "High Dose Hook Effect", repeat the test using a 10-10,000 fold dilution of the sample.

3.7.3 Stability, Storage and Shelf Life

3.7.3.1 The OneStep ABAcard® p30 Detection Test should be stored below 82° F (28° C).

3.7.3.2 The test can be stored in the sealed pouch below 82° F (28° C) until the expiration date as printed on the sealed test pouch.

3.7.3.3 DO NOT FREEZE.

3.7.3.4 Do not use the test after the expiration date.

3.7.4 Reagents and Materials Provided

3.7.4.1 Test Device (25 pieces, each individually sealed in a test pouch) - one device needed per sample tested

3.7.4.2 A dropper and a desiccant sealed inside each of the test pouches

3.7.4.3 Test Instructions

3.7.5 Equipment Required But Not Provided

3.7.5.1 Microcentrifuge

3.7.5.2 Timer

3.7.5.3 Scissors

3.7.5.4 Tweezers

3.7.5.5 Microcentrifuge tube rack

3.7.5.6 Pipettors (1000 µl and/or 200 µl)

3.7.5.7 Dissecting needle

3.7.6 Materials Required But Not Provided

3.7.6.1 Microcentrifuge tubes

3.7.6.2 Microcentrifuge tube lids

3.7.6.3 Pipette tips

3.7.7 Reagents Required But Not Provided

3.7.7.1 Known semen sample

3.7.7.2 Reagent blank

3.7.7.3 Distilled water

3.7.8 Minimum Standards and Controls

3.7.8.1 On the day of use a positive reagent control (known seminal fluid) and a negative reagent control (distilled water) must be tested to ensure that the reagents and test device are working properly. The results of this testing must be documented in the case file.

3.7.8.2 If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved as demonstrated by testing another set of positive and negative reagent controls and achieving the expected results with both controls.

3.7.8.3 A substrate control (when available) must also be tested, unless the stain is on a cotton swab, and the results of the testing documented in the case file. It is not necessary to test submitted control swabs.

3.7.9 P30 BY ONESTEP ABACARD® PROCEDURE

3.7.9.1 Cut a portion of the stain into small pieces (size based upon the substrate and the intensity of the acid phosphatase test) and place into a labeled microcentrifuge tube.

3.7.9.2 Add 200 µl of distilled water (250 µl if a sperm search is also being conducted) and cap the tube.

3.7.9.3 Allow the sample to extract at room temperature for a minimum of 2 hours. Extraction can be done overnight if desired.

3.7.9.4 Punch holes in the lid of the tube.

3.7.9.5 Place the cuttings into the lid.

3.7.9.6 Centrifuge for 5 minutes at approximately 10,000 rpm to recover the liquid.

3.7.9.7 If a microscopic sperm search is to be conducted, remove approximately 220 µl of the extract and place into a new labeled microcentrifuge tube. This aliquot will be used for the test procedure and may be stored between 2-8°C or frozen if not used immediately. The remaining extract and pellet can be used for the sperm search.

3.7.9.8 **Allow the sample to warm to room temperature** if it has been refrigerated or frozen.

3.7.9.9 Remove the device and dropper from the sealed pouch.

3.7.9.10 Add approximately 200 µl (or 8 drops with the dropper) of the sample to the sample well "S" on a labeled test device.

3.7.9.11 Record result at 10 minutes. A positive result can be seen as early as 1 minute. For negative results, one must wait for the full 10 minutes. All control samples must give the expected results before the result on an unknown sample can be called, i.e., the substrate control is negative, the reagent blank is negative, and the known semen sample is positive. A diagrammatic representation of the results is located at the end of this chapter.

3.7.9.12 Interpretation

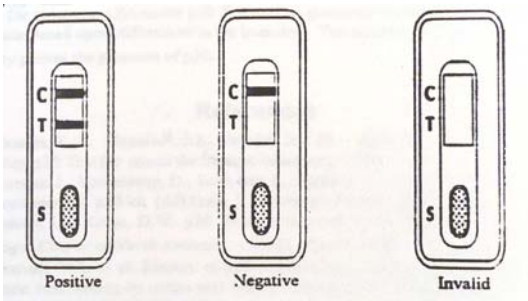
3.7.9.12.1 Positive Result = 2 pink lines, one in the test area "T" and one in the control area "C".
p30 level is at or above 4 ng/ml

3.7.9.12.2 Negative Result = 1 pink line in the control area "C".
No p30 is present above 4 ng/ml <u>OR</u> presence of "High Dose Hook Effect".

3.7.9.12.3 Invalid Result = No pink line in the control area "C".
The test is inconclusive.
Repeat the test if sufficient sample remains.

3.7.9.13 Refer to Section 3.8 for reporting results.

OneStep ABAcard® p30 TEST RESULTS DIAGRAMMATIC REPRESENTATION



Note: The diagrammatic representation of the OneStep ABAcard® p30 Test results is taken from Abacus Diagnostics, OneStep ABAcard® p30 Test For Identification of Semen, Technical Information Sheet (Revised 10/98).

3.8 Reporting Results

3.8.1 Report the results of semen testing using the statements which follow:

3.8.1.1 Positive findings

3.8.1.1.1 "Spermatozoa were identified …"

3.8.1.1.2 "A spermatozoon was identified …"

3.8.1.1.3 "Seminal fluid, but no spermatozoa, was identified …"

3.8.1.2 Negative findings

3.8.1.2.1 "No spermatozoa or seminal fluid was detected…"

3.8.1.2.2 "No seminal fluid was detected…" (This wording will also be used if only a visual exam, with or without ALS or AP, was conducted.)

3.8.1.3 Inconclusive findings

3.8.1.3.1 "Tests for seminal fluid were inconclusive…"

3.8.1.3.2 "Tests for seminal fluid were inconclusive and the stain was insufficient for further body fluid identification testing…"

♦END